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<p>(21) International Application Number: PCT/US92/09254</p> <p>(22) International Filing Date: 4 November 1992 (04.11.92)</p> <p>(30) Priority data:</p> <table><tr><td>787,271</td><td>4 November 1991 (04.11.91)</td><td>US</td></tr><tr><td>788,673</td><td>6 November 1991 (06.11.91)</td><td>US</td></tr><tr><td>891,267</td><td>29 May 1992 (29.05.92)</td><td>US</td></tr></table> <p>(71) Applicant: ADEZA BIOMEDICAL CORPORATION [US/US]; 1240 Elko Drive, Sunnyvale, CA 94089 (US).</p> <p>(72) Inventors: SENYEI, Andrew, E. ; 30551 Hilltop Way, San Juan Capistrano, CA 92675 (US). CASAL, David, C. ; 181 Center, No. 26, Mountain View, CA 94041 (US).</p>		787,271	4 November 1991 (04.11.91)	US	788,673	6 November 1991 (06.11.91)	US	891,267	29 May 1992 (29.05.92)	US	<p>(74) Agents: TERLIZZI, Laura et al.; Skjerven, Morrill, MacPherson, Franklin & Friel, 25 Metro Drive, Suite 700, San Jose, CA 95110 (US).</p> <p>(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).</p> <p>Published <i>With international search report.</i></p>	
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<p>(54) Title: SCREENING METHOD FOR IDENTIFYING WOMEN AT INCREASED RISK FOR PRETERM DELIVERY</p> <p>(57) Abstract</p> <p>The present invention provides an early, biochemical indication of increased risk of impending preterm delivery. The method comprises obtaining a cervicovaginal secretion sample from a pregnant patient after week 12 of gestation and determining the level of a local inflammatory product protein or an extracellular matrix protein in the sample. The presence of an elevated level of the selected protein in the sample indicates an increased risk of delivery. The test is a screening assay that can detect women at risk of imminent delivery, as early as two to three weeks prior to delivery.</p>												

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- 1 -

TITLE OF THE INVENTION

SCREENING METHOD FOR IDENTIFYING WOMEN
AT INCREASED RISK FOR PRETERM DELIVERY

5 BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to methods for detection of impending preterm delivery. In particular, this invention is directed to the determination of impending delivery by 10 detecting an increased level of a local inflammatory product protein or a matrix protein in a cervicovaginal secretion sample.

Description of the Prior Art

Determination of impending preterm births is critical 15 for increasing neonatal survival of preterm infants. In particular, preterm neonates account for more than half, and maybe as much as three-quarters of the morbidity and mortality of newborns without congenital anomalies.

Although tocolytic agents which can delay delivery were 20 introduced 20 to 30 years ago, there has been only a minor decrease in the incidence of preterm delivery. It has been postulated that the failure to observe a larger reduction in the incidence of preterm births is due to errors in the diagnosis of preterm labor and to the 25 patients' conditions being too advanced for tocolytic agents to successfully delay the birth.

Traditional methods of diagnosis of preterm labor have high false-negative and false-positive error rates [Friedman et al, *Am. J. Obstet. Gynecol.* 104:544 (1969)].

30 In addition, traditional methods for determining impending preterm delivery, particularly in patients with clinically intact membranes, may require subjective interpretation, may require sophisticated training or equipment [Garl et

- 2 -

al, *Obstet. Gynecol.* 60:297 (1982)] or may be invasive [Atlay et al, *Am. J. Obstet. Gynecol.* 108:933 (1970)]. An early, objective biochemical marker which indicated increased risk for preterm delivery was sought.

5 Recently, Lockwood et al [New Engl. J. Med., 325:669-674 (1991)] reported that fetal fibronectin in cervical and vaginal secretions indicates pregnancies which are at risk of imminent delivery. The authors postulate that damage to the fetal membranes may release fetal 10 fibronectin into the cervix and vagina, giving rise to the biochemical marker.

15 other markers which may be released in women with true threatened pregnancies can be used to screen those women who should be closely monitored and to provide additional information about the stage of the disease.

SUMMARY OF THE INVENTION

The present invention provides an early, biochemical indication of increased risk of impending preterm delivery. The method comprises obtaining a cervicovaginal 20 secretion sample from a pregnant patient after about week 12 of gestation and determining the level of a local inflammatory product protein or a matrix protein in the sample. The presence of an elevated level of the selected protein in the sample indicates an increased risk of 25 imminent delivery. The test is both a sensitive and specific screen for pregnancies at risk and can detect impending delivery as early as two to three weeks prior to delivery.

The test is preferably administered to women at about 30 12 weeks gestation and repeated at each perinatal visit (every two to four weeks) until at least week 37, preferably until delivery, if the test is negative. For those patients whose assay result indicates an increased 35 risk of preterm delivery, a test of the patient's fetal fibronectin level can be made to confirm the increased risk and to estimate how soon the delivery may be. In

- 3 -

addition, those patients can be carefully monitored, as for other patients at risk.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a screening assay which provides an early, biochemical indication of increased risk of preterm delivery. The method can provide an indication of impending preterm delivery as early as two to three weeks prior to delivery. This method allows early intervention in the course of preterm delivery and provides an additional factor which can indicate those pregnancies at greatest risk.

The method comprises obtaining a cervicovaginal secretion sample from the vaginal cavity or the external cervical canal from a pregnant patient after about week 12 of pregnancy and prior to about week 36 or 37 and determining the level of a local inflammatory product protein or a matrix protein in the sample. The presence of an elevated level of the protein in the sample indicates a patient who is at risk for preterm delivery.

The present method can determine impending delivery from early in gestation through week 36 or 37 deliveries. Deliveries prior to 20 weeks gestation are called spontaneous abortions rather than preterm deliveries. The present method can be used to detect spontaneous abortions (12 to 20 weeks gestation) and preterm deliveries (20 to 37 weeks gestation).

Patients to be Tested

The present method can be used on any pregnant woman following about 12 weeks gestation and prior to term pregnancies (week 36 or 37). In addition to screening any pregnant woman to determine whether delivery is imminent, the patients who should be screened are those patients with clinically intact membranes in a high risk category for preterm delivery, and preferably, all those women whose pregnancies are not sufficiently advanced to ensure

- 4 -

delivery of a healthy fetus. Ninety percent of the fetal morbidity and 100 percent of the fetal mortality associated with preterm delivery is for those fetuses delivered prior to 32 to 34 weeks gestation. Therefore,
5 32 to 34 weeks gestation is an important cutoff for the health of the fetus, and women whose pregnancies are at least about 12 weeks and prior to 34 weeks in gestation should be tested.

In addition there are a large number of factors known
10 to be associated with the risk of preterm delivery. Those factors include multiple fetus gestations; incomplete cervix; uterine anomalies; polyhydramnios; nulliparity; previous preterm rupture of membranes or preterm labor; preeclampsia; first trimester vaginal bleeding; little or
15 no antenatal care; and symptoms such as abdominal pain, low backache, passage of cervical mucus and contractions. Any pregnant woman at 12 or more weeks gestation with clinically intact membranes and having one or more risk factors for preterm delivery should be tested throughout
20 the risk period; i.e. until about week 34 to 37.

Sample

The sample is obtained in the vicinity of posterior fornix, the ectocervix or external cervical os. The sample generally comprises fluid and particulate solids,
25 and may contain vaginal or cervical mucus and other vaginal or cervical secretions. The sample is preferably removed with a swab having a dacron or other fibrous tip. Alternatively, the sample can be obtained with a suction or lavage device. Calculations to account for any
30 additional dilution of the samples collected using liquids can be performed as part of the interpretation of the assay procedure.

Following collection, the sample is transferred to a suitable container for storage and transport to a testing
35 laboratory. It is important that the sample be dispersed in a liquid which preserves proteinaceous analytes. The

- 5 -

- storage and transfer medium should minimize, preferably prevent, decline in the analyte level during storage and transport. A suitable solution for storage and transfer consists of 0.05 M Tris-HCl, pH 7.4; 0.15 M NaCl, 0.02% 5 NaN_3 , 1% BSA, 500 Kallikrein Units/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA, and is described in U.S. Patent No. 4,919,889, issued April 24, 1990. The solution is also suitable as a sample diluent solution.
- 10 Alternatively, home and office use devices for immediate processing of the sample can be used. If used, the sample is placed directly in the device and testing is performed within minutes of sample collection. In such cases, the need to stabilize the analyte is minimized and 15 any solution which facilitates performing the assay and is not detrimental to analyte stability can be used.

Analyte

Local Inflammatory Product Proteins

Local inflammatory product proteins are synthesized 20 and secreted during acute inflammation and have a protective effect that helps to maintain homeostasis in the extracellular space. The local inflammatory proteins are well known and include proteins produced by the complement-activated cellular and humoral immune response.

25 Local inflammatory product proteins include five major categories of proteins which are released as part of the local inflammatory response. Cytokines are the first category of local inflammatory product proteins. Cytokines include interleukins (particularly IL-1, IL-6 and IL-8), transforming growth factor (particularly TGF β), and tumor necrosis factor (TNF α and TNF β). Prostenooids 30 are the second category of local inflammatory product proteins. Prostenooids include prostaglandins and the leukotrienes. However, prostaglandins are preferably 35 tested following week 20 of gestation. The third category

- 6 -

of local inflammatory product proteins includes acute phase reactant proteins. Acute phase reactant proteins include fibronectin, ceruloplasmin, C-reactive protein, α_1 -anti-trypsin, α_1 -anti-chymotrypsin, α_2 -macroglobulin, 5 interferons, fibrin, serum amyloid A protein, complement (especially c3 and c4), and α_1 -acid glycoprotein. The fourth category includes the local response immunoglobulins IgA and IgM. The sixth category includes soluble factors from macrophages and monocytes, lactoferrin and lysozymes. 10 Of importance are those local inflammatory product proteins known to be produced in the reproductive tract and in connective tissue.

Extracellular Matrix Proteins

A substantial portion of the volume of a tissue is 15 extracellular space which is largely filled with an intricate network of macromolecules that comprise the extracellular matrix. The extracellular matrix is comprised of proteins and polysaccharides that are secreted locally and determine the physical properties of 20 the tissue.

As used herein, the term "extracellular matrix protein" means an adult isoform of the selected extracellular matrix protein and does not include fetal isoforms of the protein which are present during pregnancy 25 such as fetal fibronectin. Preferred extracellular matrix proteins are those of the amniotic epithelium, chorion, decidual epithelium and basal lamina. In addition, the protein is one that is preferably liberated from the maternal/decidual interface and/or the uterine cervix as a 30 result of inflammatory or proteolytic process or, alternatively is present as the result of excessive production or secretion stimulated by abnormal autocrine events at the maternal/decidual interface and/or the uterine cervix. In addition to detection of an 35 extracellular matrix protein, detection of fragments of the proteins produced by proteolytic cleavage including

- 7 -

chemical and enzymatic cleavage of the protein, is also contemplated. These fragments of the protein are the breakdown products of the protein and can include carbohydrate moieties released when the protein is
5 degraded.

There are two functional types of extracellular matrix proteins, structural proteins and adhesive proteins. In addition, the extracellular matrix proteins include secretory proteins which are secreted in large
10 amounts by the neighboring cells at the site. The structural extracellular matrix proteins include collagen and elastin. The adhesive extracellular matrix proteins include fibronectin, tenascin, vitronectin, and laminin. The secretory proteins include insulin-like growth factor
15 binding protein (IGFBP) (particularly IGFBP-1), elastin receptor protein, tropoelastin, tropocollagen, decorin (dermatan sulfate), mucin and CA125. Degradation products of the proteins include desmosine (a breakdown product of elastin), sialic acid (a carbohydrate moiety released by
20 the breakdown of many of these proteins) and peptide fragments.

Assay Procedure

The selected local inflammatory product protein or extracellular matrix protein is assayed by any procedure
25 which can determine the presence of a threshold quantity of the selected protein in the sample. Immunoassays are preferred. The antibody affinity required for detection of the protein (the analyte for the assay) using a particular immunoassay method will not differ from that
30 required to detect other polypeptide analytes. Furthermore, in many cases, proteolytic cleavage of the selected local inflammatory product protein or extracellular matrix protein does not effect the assay. That is, many epitopes of these proteins, particularly
35 large extracellular matrix proteins, remain intact following proteolysis. In such cases, an antibody for the

- 8 -

epitope does not distinguish between intact forms of the protein and protein fragments. However, in cases where proteolysis does eliminate the epitope recognized by a selected antibody, an antibody that binds to an epitope 5 that remains intact can be selected.

In some cases, proteolysis can create an epitope that is not present on the intact protein and is only present on the resultant fragment. In such cases, the use of antibodies specific for such epitopes to detect the 10 resulting fragments is also contemplated herein.

Furthermore, determination that a fragment of an extracellular matrix protein is present is also contemplated. In cases where the proteolysis produces an epitope not present on the intact protein, the presence a 15 threshold amount of the fragment can be detected using an antibody to the epitope. In addition, where one or more epitopes are intact, the use of a Western blot assay that determines the size of the proteins to which the selected antibody binds can be used to detect the presence of a 20 fragment of the protein.

Anti-analyte antibodies can be produced by a number of methods. Polyclonal antibodies can be induced by administering an immunogenic composition comprising the selected local inflammatory product protein to a host 25 animal. Preparation of immunogenic compositions of a protein may vary depending on the host animal and the protein and is well known. For example, the analyte protein or an antigenic portion thereof can be conjugated to an immunogenic substance such as KLH or BSA or provided 30 in an adjuvant or the like. The induced antibodies can be tested to determine whether the composition is for the analyte protein. If a polyclonal antibody composition does not provide the desired specificity, the antibodies 35 can be purified to enhance specificity by a variety of conventional methods. For example, the composition can be purified to reduce binding to other substances by contacting the composition with the analyte protein

- 9 -

affixed to a solid substrate. Those antibodies which bind to the substrate are retained. Purification techniques using antigens affixed to a variety of solid substrates such as affinity chromatography materials including

5 Sephadex, Sepharose and the like are well known.

Monoclonal analyte-specific antibodies can also be prepared by conventional methods. A mouse can be injected with an immunogenic composition comprising the analyte protein, and spleen cells obtained. Those spleen cells
10 can be fused with a fusion partner to prepare hybridomas. Antibodies secreted by the hybridomas can be screened to select a hybridoma wherein the antibodies react with the analyte and exhibit substantially no reaction with the other proteins which may be present in a sample.
15 Hybridomas that produce antibodies of the desired specificity are cultured by standard techniques. Hybridoma preparation techniques and culture methods are well known and constitute no part of the present invention.

20 An exemplary preparation of polyclonal anti-fibronectin antibodies is described in the examples. Antibody preparation and purification methods are described in a number of publications including Tijssen,
25 P. Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theories of Enzyme Immunoassays New York: Elsevier (1985), for example.

A number of different types of immunoassays are well known using a variety of protocols and labels. The assay conditions and reagents may be any of a variety found in
30 the prior art. The assay may be heterogeneous or homogeneous, conveniently a sandwich assay.

The assay usually employs solid phase-affixed anti-analyte antibodies. The antibodies may be polyclonal or monoclonal. The solid phase-affixed antibodies are
35 combined with the sample. Binding between the antibodies and sample can be determined in a number of ways. Complex formation can be determined by use of soluble antibodies

- 10 -

- specific for the analyte protein. The antibodies can be labeled directly or can be detected using labeled second antibodies specific for the species of the soluble antibodies. Various labels include radionuclides, enzymes, fluorescers, colloidal metals or the like.
- 5 Conveniently, the assay will be a quantitative enzyme-linked immunosorbent assay (ELISA) in which antibodies specific for the analyte protein are used as the solid phase-affixed and enzyme-labeled, soluble antibodies.
- 10 Alternatively, the assay can be based on competitive inhibition, where analyte in the sample competes with a known amount of analyte or analyte analog for a predetermined amount of anti-analyte antibody. For example, in an assay for fibronectin, any fibronectin
- 15 present in the sample can compete with a known amount of the labeled fibronectin or a fibronectin analogue for antibody binding sites. The amount of labeled fibronectin affixed to the solid phase or remaining in solution can be determined.
- 20 In another preferred embodiment, the assay is a homogeneous immunoassay in which antibodies specific for the analyte are used as the solid phase-affixed and colloidal metal-labeled, soluble antibodies. Appropriate dilution of the conjugate can be performed to detect the
- 25 selected threshold level of the analyte as a positive sample.

Threshold Value

- The amount of a local inflammatory product protein or a matrix protein in the sample is significantly elevated over the level for pregnant women at the same stage of gestation with normal pregnancies. Preferably, the selected threshold value is two standard deviations above normal.

- It is difficult to quantify proteins in vaginal swab samples for two reasons. The first is that the amount of fluid collected by the swab varies. The second is the

- 11 -

total volume of secretions present in the cervicovaginal region varies. Therefore, any measurement of protein concentration in a particular sample is only a semi-quantitative indication of the total amount of the protein
5 in the cervicovaginal region. Therefore, it is desirable that patients with samples near the threshold value be retested in a follow up visit.

The threshold value that correlates with risk of preterm delivery varies for the local inflammatory product protein or matrix protein selected for analysis. In
10 addition, the value can vary with the stage of gestation of the patient. To determine the threshold value for a particular local inflammatory product protein or matrix protein, a prospective-retrospective study is performed to
15 determine the threshold value for one or more selected local inflammatory product protein(s) and or matrix protein(s). These studies are well known and can be performed in a number of ways. An exemplary preferred method is described below.

20 In a preferred study, at least ten samples each from about 2,000 pregnant women are obtained. The sample collections are approximately evenly spaced beginning at about week 8 throughout gestation. The samples are collected at least every three to four weeks. Preferably,
25 the samples are collected every two weeks.

About 5 to 10% of pregnant women deliver preterm with preterm labor and without preterm rupture of membranes. Therefore, of the 2,000 women in the study, about 50 women
30 who deliver early will have preterm labor without preterm rupture of membranes.

When those women are identified, matched controls are selected. Those controls are matched for well known risk factors such as age, parity, obstetrical history, and socio-economic factors. Then samples from the controls
35 are matched for sample time and stage of gestation. The level of the local inflammatory product protein(s) and matrix protein(s) is then determined for the samples.

- 12 -

Receiver-operator statistics to determine sensitivity and specificity are then performed. The statistics can be used to select for screening so that few women at risk will be falsely negative in the test but a comfortable level of false positives are also achieved. The threshold is preferably adjusted to detect substantially all women at risk since other criteria can be considered to further assess the risk prior to institution of any treatment.

A study demonstrating that a threshold value of total fibronectin in the range of 600 to 750 ng/ml of sample for women from about 20 weeks gestation to delivery is an indication of risk is described in detail in the examples.

Interpretation of Assay Result

Elevated levels of a local inflammatory product protein or a matrix protein indicate increased risk of preterm delivery. As explained in detail in the examples, an assay based on total fibronectin is sensitive and specific. In addition, the assay has a high negative predictive value. That means that a large percentage of patients who delivered early had an elevated fibronectin value. Since the test successfully detects a large percentage of patients who deliver early, the test is an effective screening procedure for women at risk of a preterm delivery who do not have any other risk indicators.

The test can be administered to any pregnant woman following about 12 weeks gestation until delivery, or at least until the risk of premature delivery (i.e. until about week 37) ceases. Preferably, it is administered to all women with any known risk factor following 12 weeks gestation until delivery.

If the local inflammatory product protein or matrix protein test is positive (above the threshold value), the patient is preferably tested for the presence of fetal fibronectin in her cervicovaginal secretions. If fetal fibronectin is present in the secretions, the patient is

- 13 -

likely to deliver in two to three days. Measures to determine or enhance fetal lung maturity can be undertaken. If the fetal fibronectin assay is negative, the patient should be carefully monitored and repeated evaluations of the patient's fetal fibronectin levels should be performed on subsequent visits. In general, patients at risk for preterm delivery are examined every two weeks from about 22 to 36 weeks, rather than every four weeks as for patients in a low risk category.

If the local inflammatory product protein or matrix protein test is negative, the test is preferably repeated on each subsequent antenatal visit until either the test is positive or the patient reaches term.

This invention is further illustrated by the following specific but non-limiting examples. Temperatures are given in degrees Centigrade and concentrations as weight percent unless otherwise specified. Procedures which are constructively reduced to practice are described in the present tense, and procedures which have been carried out in the laboratory are set forth in the past tense.

EXAMPLE 1

Quantitation of Total Fibronectin in a Vaginal Swab Sample

An immunoassay to determine total fibronectin in a vaginal sample used the reagents and procedures described below.

Preparation of Polyclonal Anti-Human Fibronectin Antibody

Human plasma fibronectin was purified from human plasma as described by Engvall and Ruoslahti, *Int. J. Cancer* 20:1-5 (1977). The anti-human plasma fibronectin antibodies were elicited in goats using the immunization techniques and schedules described in the literature, e.g., Stollar, *Meth. Enzym.* 70:70 (1980), immunizing the goats with the human plasma fibronectin antigen. The

- 14 -

antiserum was screened in a solid phase assay similar to that used for monoclonal antibodies, e.g., as described by Lange et al, *Clin. Exp. Immunol.* 25:191 (1976) and Pisetsky et al, *J. Immun. Meth.* 41:187 (1981).

5 The IgG fraction of the antiserum was purified further by affinity chromatography using CNBr-Sepharose 4B (Pharmacia Fine Chemicals) to which has been coupled human plasma fibronectin according to the method recommended by the manufacturer (AFFINITY CHROMATOGRAPHY, Pharmacia Fine 10 Chemicals Catalogue 1990), pp 15-18.

Briefly, the column was equilibrated with from 2 to 3 volumes of buffer (0.01 M PBS, pH 7.2), and the anti-human fibronectin antibody-containing solution was then applied to the column. The absorbency of the effluent was 15 monitored at 280 nm until protein no longer passed from the column. The column was then washed with equilibration buffer until a baseline absorbance at 280 nm was obtained.

20 The immunoaffinity bound anti-human plasma fibronectin antibody was eluted with 0.1 M glycine buffer, pH 2.5. Peak protein fractions were collected, pooled and dialyzed against 0.01 M PBS, pH 7.2, for 24-36 hr at 4°C with multiple buffer changes.

25 The above procedure was repeated to immunize rabbits with human plasma fibronectin and to purify the resultant polyclonal anti-human fibronectin antibodies.

Preparation of Anti-Fibronectin Antibody-Coated Microtiter Plate

30 Goat anti-human plasma fibronectin prepared as described above was diluted to 10 µg/ml in 0.05 M carbonate buffer, pH 9.6. 100 µl was dispersed into each well of a polystyrene microtiter plate such as supplied by Costar, Nunc, or Dynatech. The plate was covered and incubated 2 to 4 hr at room temperature or 4°C overnight. The plate was washed 3 to 4 times with Wash Buffer (0.02 M Tris HCl, 0.015 M NaCl, 0.05% TWEEN-20), filling and emptying the wells completely with each use. The plate 35

- 15 -

- was then blocked by dispersing 200 μ l of a blocking/stabilizing solution (4% sucrose, 1% mannitol, 0.01 M PBS, 1% BSA, 0.02% NaN₃, pH 7.4) into each well and incubating for 30 minutes to 2 hrs at room temperature.
- 5 The wells were then aspirated to dryness, the plate was packaged in an air-tight container with a desiccant pouch, and stored at 4°C until needed. The wells were present as eight well strips.

Preparation of Enzyme Labeled Anti-(fibronectin) Antibody

- 10 Anti-human plasma fibronectin antibody prepared as described above was conjugated with alkaline phosphatase following the one-step glutaraldehyde procedure of Avrameas, *Immunochem.* 6:43 (1969).

Assay Reagents

- 15 The assay was performed using the following additional reagents. The stock antibody conjugate was appropriately diluted in conjugate diluent (0.05 M Tris Buffer pH 7.2, 2% D-Sorbitol, 2% BSA, 0.1% Sodium Azide, 0.01% Tween-20, 1 mM Magnesium Chloride, and 0.1% Zinc Chloride) and 10 ml placed in a polyethylene dropper bottle container.

The enzyme substrate (10 ml in a polyethylene dropper bottle container) was phenolphthalein monophosphate (1 mg/ml) dissolved in 0.4 M aminomethylpropanediol buffer, pH 10 with 0.1 mM magnesium chloride and 0.2% sodium azide.

- The fibronectin calibration solutions were plasma fibronectin (fibronectin from human serum purchased from Boehringer Mannheim, Indianapolis, IN; Catalogue No. 30 1050407) diluted to a concentration of 0.0, 0.01, 0.05 and 0.25 μ g/ml in sample diluent solution (0.05 M Tris buffer pH 7.4, 1% bovine serum albumin (BSA), 0.15 M sodium chloride, 0.02% Sodium Azide, 5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 500 Kallikrein Units/ml of

- 16 -

Aprotinin). This sample diluent solution is described in U.S. Patent No. 4,919,889 to Jones et al, issued April 24, 1990, which patent is incorporated herein by reference in its entirety. The negative control was the sample diluent 5 solution used for the positive control without fibronectin.

The rinse buffer (10 ml in a polyethylene dropper bottle container) was a 50X concentrate containing 1.0 M Tris buffer pH 7.4, 4.0 M sodium chloride, 2.5% Tween-20, 10 and 1% sodium azide. The rinse buffer was diluted with water to a final concentration of 0.02 M Tris, 0.08 M sodium chloride, 0.05% Tween-20, and 0.02% sodium azide for use in the assay.

In addition, 5 μ pore size polyethylene sample 15 filters (Porex Technologies, Fairburn, Georgia) were used to filter the samples prior to assay. All of the dropper bottles used to perform the assay were polyethylene bottles designed to dispense approximately 50 μ l drops of the reagent. All of the assay steps performed following 20 sample collection utilized the reagents and materials described above.

Assay Procedure

The assay was performed as follows. All samples were collected in the vicinity of the posterior fornix or 25 cervical os using dacron swabs. Swab samples were immersed in 1.0 ml of sample diluent in a collection vial. The swabs were removed from the solution leaving as much liquid as possible in the collection tube. The samples were incubated at 37°C along with the controls for 15 30 minutes prior to the assay, either before or after filtration. A sample filter was snapped in place on each sample tube. The 8-well strips were snapped into place in a strip holder. The holder had the alphanumeric indications of the 12 columns and eight rows of standard 35 microtiter plates. Duplicate 100 μ l aliquots of each sample and the positive and negative controls were placed

- 17 -

in separate wells of the microtiter strip and incubated for 1 hour at room temperature.

Following incubation, samples and controls were aspirated from the wells. Wells were washed three times 5 with diluted wash buffer (1X). Following washing, 100 μ l of enzyme-antibody conjugate was added to each well and incubated for 30 minutes at room temperature. The wells were aspirated and washed as described above. Following washing, 100 μ l of enzyme substrate was added to each well 10 and incubated for 30 minutes at room temperature.

Following the incubation, the plates were gently agitated by hand or with an orbital shaker to mix the well contents. The frame of strips was placed in an ELISA plate reader. The absorbance of each well at 550 nm was 15 determined. The average absorbance of the duplicate wells for each sample and control was calculated. The total fibronectin concentration for the samples was calculated by preparing a standard curve and estimating that the samples were diluted to about one-tenth of their original 20 concentration (collection of about 0.1 ml of sample combined with 1.0 ml of diluent).

EXAMPLE 2

Detection of a Threshold Amount of Total Fibronectin in a Vaginal Swab Sample

25 In another preferred embodiment, an assay kit to detect a threshold amount of fibronectin includes the following components. This kit is designed to be used to perform a rapid, bedside or doctor's office assay.

1. an assay device comprising a plastic housing and 30 containing:
 - (a) a porous nylon membrane to which is bound an anti-fibronectin antibody;
 - (b) a flow control membrane system; and
 - (c) an absorbent layer
- 35 2. a colloidal gold-labeled goat anti-fibronectin antibody conjugate in a protein matrix

- 18 -

3. conjugate reconstitution buffer
4. a wash solution
5. a sterile, dacron sample collection swab

5 The membrane device is prepared by the following procedure. Approximately 2 μ l of the polyclonal anti-fibronectin antibody prepared as described in Example 1 is applied to a membrane surface (1.2 μ nylon, Biodyne-A, Pall) in a pH 6, 0.01 M phosphate buffered saline (PBS), 0.1 M citrate buffer containing 0.5 mg/ml BSA. A 10 procedural control consisting of human plasma fibronectin purified as described in Example 1 in the same buffer is also applied to a discrete region of the membrane. After the membrane has air dried, a blocking reagent of PBS-buffered, 0.5% nonfat dry milk is added to the membrane. 15 The excess blocking reagent is removed after at least about 20 minutes.

15 The membrane-holding device (Target Device, V-Tech, Pomona, CA) is assembled with a second porous layer (0.45 μ low protein-binding nylon, LoProdyne, Pall) beneath the 20 antibody-bearing membrane (in the direction of sample application) for controlling the flow of sample solution from the assay membrane to the absorbent layer. The two porous membranes are then placed over an absorbent porous polyethylene layer having a capacity of greater than 1.5 25 ml (Chromex, Brooklyn, NY) and enclosed in the device. The device is packaged individually in a sealed plastic bag containing desiccant.

30 The colloidal gold was prepared by the reduction of 0.01% tetrachloroauric acid with 0.16% sodium citrate in a manner which produces approximately 30 nm particles. Briefly, the two solutions are heated separately to 90°C. The reducing solution is added to the gold solution while vigorously stirring. The combined solution is boiled (100°C) for at least 10 minutes.

35 Affinity purified goat anti-fibronectin antibody (prepared as described in Example 1) was bound to the

- 19 -

colloidal gold by adsorption. Briefly, the colloidal gold solution prepared above was combined with the antibody (5-10 µg/ml) in water. Following conjugation, the conjugate solution is stabilized by the addition of 5% BSA and 5% polyvinylpyrrolidone (final concentration).

The stock conjugate was concentrated approximately 10- to 12-fold by ultrafiltration using a hollow fiber filter. The concentrated conjugate was diluted to an appropriate level in 15 mM Tris, 2% BSA, 0.1% Tween 20, 0.2% polyethylene glycol, 8% polyvinylpyrrolidone and 0.04% thimerosal. An appropriate concentration is determined by using a range of dilutions in a sample assay procedure as described below and determining the dilution which produces the best result. This titration procedure is used to set the threshold detection level for total fibronectin at 750 ng/ml.

The selected conjugate dilution is placed in polyethylene sample collection tubes and lyophilized. The tubes are fitted with 2 µ pore size polyethylene sample filters (Porex Technologies, Fairburn, Georgia) during the lyophilization process. The lyophilized conjugate is individually packaged in a foil pouch with desiccant.

The conjugate reconstitution buffer is 100 mM sodium acetate. This buffer is packaged as a unit dose in a 1 ml disposable tube. The wash solution is water packaged as a unit dose in a disposable tube. The kit additionally contains an individually packaged sterile dacron swab and a procedural summary card.

The assay was performed as follows.

- 30 1. Before collecting the sample, remove the plastic tube containing gold conjugate from the foil pouch, remove the dropper tip and add the entire contents of the tube containing the conjugate reconstitution buffer.
2. Collect the sample with the swab provided. During a sterile speculum examination, insert the swab into the posterior fornix of the vagina, twirl for approximately 10 seconds to absorb fluid.

- 20 -

Immediately proceed to perform the test. Samples may not be stored for later testing. Place the swab in the gold conjugate solution and mix rapidly with an up and down motion for 10 to 15 seconds.

- 5 3. Remove as much liquid as possible from the swab by rolling the tip on the inside of the tube. Dispose of the swab in a manner consistent with handling potentially infectious materials.
- 10 4. Replace the dropper tip on the plastic tube and immediately dispense the entire volume of diluted filtered sample onto the surface of the membrane device.
- 15 5. After the sample liquid has been absorbed into the membrane surface, add a few drops of wash solution and observe the results.
- 20 6. A negative result is indicated by a red color in the procedural control area of the membrane only. A positive result is indicated by a pink or red spot in the test zone of the membrane as well as in the control zone.

EXAMPLE 3

Total Fibronectin Levels in Preterm Patients

In an effort to evaluate cervicovaginal expression of total fibronectin as a screen for preterm delivery (preterm delivery), 73 asymptomatic women with elevated risk for preterm delivery were identified and followed longitudinally. Factors which defined these women as having higher risk included pregnancy with twins, uterine anomalies, previous preterm labor and previous preterm delivery. Vaginal specimens were obtained from these women at 1 to 2 week intervals between 24 and 34 weeks gestation. Vaginal secretions were collected from the ectocervical region of the external cervical os and the posterior fornix of the vagina using separate dacron swabs.

- 21 -

The total fibronectin concentration of vaginal secretion samples in this study was determined as described in Example 1. The average concentration of total fibronectin in cervicovaginal secretions was 5 4.49 ± 0.50 $\mu\text{g}/\text{ml}$ (mean \pm SEM) for women with uncomplicated pregnancies prior to 22 weeks gestation. The concentration of total fibronectin exceeded 750 ng/ml in 64.8% (250/386) of these women. After 22 weeks gestation the average concentration of total fibronectin was 10 0.99 ± 0.50 and 22.2% (4/18) had values greater than 750 ng/ml.

From those values, a sample was determined to be positive if its total fibronectin concentration exceeded 750 ng/ml. A single positive sample was the minimal 15 requirement for definition of a positive patient. The sample results for all women were accumulated and compared to gestational age at the time of delivery. Delivery prior to 37 weeks and 0 days was defined as preterm while a pregnancy exceeding 37 days and 0 days was defined as 20 term.

Of the 73 patients enrolled in this evaluation, 24 delivered prematurely and 49 delivered at term. The relationship of total fibronectin results and clinical outcome is shown in Table 1. In the analyses, sensitivity 25 means the number of true positive test results divided by the total number of women with the condition; i.e., the number of true positive test results divided by the sum of the number of true positive and false negative test results). Specificity means the number of true negative 30 test results divided by the total number of women without the condition; i.e., the number of true negative test results divided by the sum of the number of true negative and false positive test results. Positive predictive value (PV +) means the number of true positive test 35 results divided by the total number of samples which tested positive. Negative predictive value (PV -) means the number of true negative test results divided by the

- 22 -

total number of samples which tested negative. Sensitivity, specificity, positive predictive value and negative predictive value are based on detection of preterm delivery (PTD), rather than term delivery (TD) in 5 this analysis.

	FN +	FN -	
PTD	19	5	24
TD	24	25	49
	43	30	73

Sensitivity = 82.8%
Specificity = 51.0%
PV + = 44.2%
PV - = 83.3%
Relative Risk = 2.65, p<0.007
Mantel-Haenszel χ^2 = 5.98, p<0.014

15 At least one total fibronectin sample exceeded 750 ng/ml in 19 of 24 patients (sensitivity=82.8%) delivering prematurely and 24 of 49 patients delivering at term (specificity=51.0%). Alternatively, the predictive value of a positive test was 44.2% while the predictive value of a negative test was 83.3%. The relative risk associated with a positive test (relative to a negative test) was 2.65 which is significantly different from 1.00 (Null Hypothesis (H_0): Relative Risk=1.00) with a p value of less than 0.007. Moreover, the distribution of positive 20 results was associated with preterm delivery as shown by the Mantel-Haenszel χ^2 test statistic of 5.98 (p<0.014). 25

The results of the study demonstrate that the test was both a sensitive and specific screening assay in that most women who deliver early are detected by the test.

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EXAMPLE 4
Total Fibronectin Levels
in Weeks 12 to 22

35 Total fibronectin concentrations were determined in specimens of cervicovaginal secretions obtained from women with apparently uncomplicated pregnancies between 12 weeks gestation and term as described in Example 1. Average concentrations of total fibronectin were determined at

- 23 -

each gestational age.

Total fibronectin concentration was highest at 12 weeks gestation and gradually decreased until week 22. From week 22 of gestation until near term, total fibronectin concentration rarely exceeded 500 ng/ml. As gestational age advanced beyond 34 weeks, total fibronectin concentrations gradually increased presaging delivery. The gestational age dependency of total fibronectin concentration was accurately predicted by a series of second order polynomial regression equations calculated using estimated gestational age at sampling and total fibronectin concentration as the independent and dependent variables, respectively.

The predicted concentrations of total fibronectin ($\mu\text{g}/\text{ml}$) at each week of gestation are shown in the following table.

	<u>Gestational Age FN1</u>	<u>Pred [Total FN]</u>	<u>Gestational Age</u>	<u>Pred [Total</u>
20	12	6.30	25	0.10
	13	6.20	26	0.06
	14	5.94	27	0.03
	15	5.51	28	0.04
	16	4.92	29	0.07
	17	4.16	30	0.13
25	18	3.24	31	0.22
	19	2.16	32	0.33
	20	0.91	33	0.47
	21	0.57	34	0.64
	22	0.42	35	0.83
30	23	0.29	36	1.06
	24	0.18	37	1.31
			38	1.58

- 24 -

WHAT IS CLAIMED IS:

1. A method for determining an early indication of increased risk of preterm delivery comprising
 - a) obtaining a secretion sample from the vaginal cavity or the cervical canal from a pregnant patient after week 12 of pregnancy; and
 - b) determining the level of a protein selected from the group consisting of a cytokine, a leukotriene, collagenase, plasminogen activators, an acute phase reactant protein, IgA, IgM, soluble factors from macrophages and monocytes, lactoferrin, lysozymes, and an extracellular matrix protein or a breakdown product thereof in the sample, the presence of an elevated level of said protein in the sample indicating an increased risk of delivery.
- 20 2. The method of Claim 1 wherein the protein is a cytokine selected from the group consisting of an interleukin, a transforming growth factor and a tumor necrosis factor.
- 25 3. The method of Claim 1 wherein the protein is an acute phase reactant protein selected from the group consisting of fibronectin, ceruloplasmin, α_1 -anti-trypsin, α_1 -anti-chymotrypsin, α_2 -macroglobulin, interferons, fibrin, serum amyloid A protein, complement, and α_1 -acid glycoprotein.
- 30 4. The method of Claim 1 wherein the protein is an extracellular matrix protein and the extracellular matrix protein is an adhesive protein.
- 35 5. The method of Claim 4 wherein the adhesive protein is selected from the group consisting of fibronectin, tenascin, vitronectin, and

- 25 -

- 5 laminin.

6. The method of Claim 1 wherein the protein is an extracellular matrix protein and the extracellular matrix protein is a structural protein.

10 7. The method of Claim 6 wherein the structural protein is selected from the group consisting of collagen and elastin.

15 8. The method of Claim 1 wherein the protein is an extracellular matrix protein and the extracellular matrix protein is a secretory protein.

20 9. The method of Claim 8 wherein the secretory protein is selected from the group consisting of insulin-like growth factor binding protein, elastin receptor protein, tropoelastin, tropocollagen, decorin, mucin and CA125.

25 10. The method of Claim 1 wherein the protein is a breakdown product of an extracellular matrix protein.

30 11. The method of Claim 10 wherein the breakdown product is selected from the group consisting of desmosine and sialic acid.

35 12. The method of Claim 10 wherein the breakdown product is peptide fragment.

13. The method of Claim 1 wherein the sample is removed from the posterior fornix.

14. The method of Claim 1 wherein the sample is obtained from the cervical os.

15. The method of Claim 1 wherein the sample is obtained from the patient does not have an elevated level of the protein and another sample from the patient is assayed for the protein at least two weeks later.

16. The method of Claim 15 wherein a sample from the patient is obtained and assayed for the presence of the protein at intervals of about 4

- 26 -

- weeks until the patient delivers or a positive sample is obtained.
- 5 17. The method of Claim 1 wherein the sample has an elevated level of the protein and a sample from the patient is assayed for the presence of fetal fibronectin.
- 10 18. The method of Claim 17 wherein the sample does not contain fetal fibronectin and another sample from the patient is assayed for fetal fibronectin at least one week later.
- 15 19. The method of Claim 18 wherein a sample from the patient is obtained and assayed for the presence of fetal fibronectin at two week intervals until the patient delivers or a positive fetal fibronectin sample is obtained.
- 20 20. The method of Claim 1 comprising the steps of
a) contacting the sample with an antibody specific for the protein for a time sufficient for antigen-antibody binding;
and
b) determining the amount of binding.
- 25 21. A method for determining an early indication of increased risk of preterm delivery comprising
a) obtaining a secretion sample from the vaginal cavity or the cervical canal from a pregnant patient after week 20 of pregnancy; and
b) determining the level of a prostaglandin after 20 weeks gestation in the sample, the presence of an elevated level of said prostaglandin in the sample indicating an increased risk of delivery.
- 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09254

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/68, 33/88
US CL :435/4; 436/65, 86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4; 436/65, 86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 104, issued 1986 Mohamed et al. "Enzyme activities in amniotic fluid and maternal blood in sheep, before and after induced fetal death and abortion", page 383, col. 1, see abstract No. 4137p Br. Vet. J. 1985, 141(5), 498-506 (Eng.).	1-20
Y	Chemical Abstracts, Volume 102, issued 1985 Mohamed et al "Enzyme activities in amniotic fluid and maternal blood in cattle before and after induced fetal death and abortion." see page 443, column 2, abstract No. 202100t Br. Vet. J. 1985, 141(1), 49-59 (Eng.).	1-20
Y	Chemical Abstracts, Volume 96, issued 1982 Seifert et al "Radioimmunoassay for the detection of primary prostaglandins in the decidua." see page 197, col. 2 abstract No. 136771s Acta Univ Palacki Olomuc., Fac. Med. 1981, 101, 32-7 (Ger)	21
Y	Grudzinski et al, Reprod. Perinat. Med. 1985 "Placental and Fetal Protein and Hormone Measurements in Early Pregnancy Failure" pages 153-156. See all.	1-20
Y	Am. J. Obstet. Gynecol. Vol. 154 No. 1 issue January 1986, McGregor et al., "Protease production by microorganisms associated with reproductive tract infection" pages 109-114. See all.	1-20
Y	Asia-Oceania J. Obstet. Gynecol., Vol. 14, No.3, issues September 1988, Kanayam et al. "The Role of Human Neutrophil Elastase in the Premature Rupture of Membranes", pages 389-397. See all.	1-20

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search Date of mailing of the international search report

19 JANUARY 1993

08 FEB 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09254

B. FIELDS SEARCHED

Documentation other than minimum documentation that are included in the fields searched:

Chemical Abstracts 11th Collective Index General Subjects, Vol. 96-105, 1982-1986 "Abortion", "Pregnancy".